Eicosapentaenoic acid (EPA) induces peroxisome proliferator-activated receptors and ameliorates experimental autoimmune encephalomyelitis

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ABSTRACT

Eicosapentaenoic acid (EPA), one of the n−3 polyunsaturated fatty acids, is a neuroprotective lipid with anti-inflammatory properties. We investigated the possible therapeutic effect of EPA on experimental autoimmune encephalomyelitis (EAE). EAE mice were fed a diet with or without EPA. The clinical EAE scores of the EPA-fed mice were significantly lower than those of the non-EPA mice. In the EPA-treated mice, IFN-γ and IL-17 productions were remarkably inhibited and the expression levels of peroxisome proliferator-activated receptors were significantly enhanced in the CNS-infiltrating CD4 T cells. Thus EPA shows promise as a potential new therapeutic agent against multiple sclerosis.

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1. Introduction

Among the essential n−3 polyunsaturated fatty acids (PUFAs) which fish oils contain, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to provide clinical benefits to systemic arteriosclerosis such as cardiovascular disease and ischemic stroke. These n−3 PUFAs are notable not only for their lipid-lowering effects but for their anti-inflammatory effects. However, some kinds of eicosanoids produced from arachidonic acid (AA) in the n−6 PUFAs promote inflammatory and prothrombotic activities. Supplementation with n−3 PUFAs reduced T cell numbers and transcriptional levels of IL-6 in plaques of the human carotid artery and in a mouse model of atherosclerosis (Wang et al., 2009; Cawood et al., 2010). In ApoE-deficient mice, EPA supplementation suppressed the development of atherosclerosis by suppressing the expression of adhesion molecules in endothelial cells and of matrix metalloproteinase-2 (MMP-2) and MMP-9 in macrophages though a peroxisome proliferator-activated receptor (PPAR) α-dependent pathway (Matsumoto et al., 2008). Additionally, n−3 PUFAs augmented adiponectin, an anti-inflammatory adipokine, in a PPARγ-dependent manner and improved insulin resistance (Banga et al., 2009; Tishinsky et al., 2011), and n−3 PUFA gave rise to a family of anti-inflammatory mediators termed resolvins via trans-cellular biosynthesis (Schwab et al., 2007; Haworth et al., 2008). Therefore, it is now widely accepted that ingesting n−3 PUFAs leads to the suppression of inflammatory responses.

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), accompanied by multiple foci of inflammatory lesions. MS is thought to have an autoimmune pathogenesis, involving autoimmune CD4 T cells reactive to myelin antigens (Sospedra and Martin, 2005). Development of the CNS inflammation is triggered by pro-inflammatory cytokines produced by autoimmune CD4 T cells, which penetrate into the CNS parenchyma after being activated in the periphery (Hickey et al., 1991; Kawakami et al., 2005). Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for MS (Gold et al., 2006) that can be induced by active immunization with myelin antigens or by a passive transfer of CD4 T cells specific to myelin antigens in the laboratory. Recently the expansion of IL-17-producing auto-reactive CD4 T (Th17) cells in the CNS was detected using an EAE model (Langrish et al., 2005). Soon after, the existence of Th17 cells was also detected in histological analysis of MS brains (Tzartos et al., 2008). Because clinical use of IFN-γ treatment for MS actually worsened the disease (Panitch et al., 1987) and both IFN-γ-producing CD4 T clones (Th1) and Th17 clones specific for myelin antigens can induce EAE by an adoptive transfer model, it is accepted that both Th1 and Th17 cells have crucial roles in the pathogenesis of MS.

In the present study, we employed EPA to clarify the anti-inflammatory effect and therapeutic potential of EPA in MS. Here, we show that the administration of ultra-purified EPA markedly inhibited the development of EAE and almost completely blocked the anti-inflammatory cytokine production in the CNS-infiltrating CD4 T
cells. Interestingly, EPA also gave rise to all types (α, β and γ) of PPARs abundantly in the CNS-infiltrating CD4 T cells. Thus, through an effective induction of PPARs in the CNS-infiltrating CD4 T cells, EPA can be another therapeutic option in MS as well as in metabolic diseases.

2. Materials and methods

2.1. Animals

C57BL/6 (B6) female mice were purchased from CLEA Japan Inc. Mice were fed with a fish-oil-free diet (Funabashi Farm, Japan) without or with 5% (w/w) eicosapentaenoic acid ester supplementation 2 weeks before the EAE induction. To prevent the oxidation of EPA, diets were changed freshly every day and contact with metal was avoided. EAE was induced by a subcutaneous immunization with 100 μg of MOG35–55 mixed with 1 mg heat-killed Mycobacterium tuberculosis H37RA emulsified in Freund’s adjuvant (CFA). Two hundred ng of pertussis toxin (PT) was injected intraperitoneally on days 0 and 2 after immunization. Clinical signs were scored daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, partial hind limb paralysis; 4, total hind limb paralysis; and 5, fore and hind limb paralysis. For conventional histological analysis of EAE, paraffin-embedded spinal cords were stained with either hematoxylin–eosin (HE) or luxol fast blue (LFB).

2.2. Lymphocyte isolation

Mice were sacrificed after the EAE induction. Lymphocytes were isolated from the spleen (SPL) and the brain/spinal cord of EAE mice by Percoll density-gradient centrifugation. CD4 T cells were separated by an AutoMACS cell purification system (Miltenyi Biotec, Cologne, Germany) with a mouse CD4 T cell isolation kit II (Miltenyi). Total RNA was isolated from CD4 T cells or mononuclear cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany)

2.3. Quantitative RT-PCR

DNase-treated total RNAs were processed for cDNA synthesis by using random hexamer primers and a PrimeScript® RT reagent kit with gDNA Eraser (Perfect Real Time; Takara Bio). cDNAs were amplified by PCR on a Rotor-Gene PCR Cycler (QIAGEN) by using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio). Values for each gene were normalized to those of the housekeeping gene β-actin to adjust for variations between different samples. Primers for amplifying mouse PPARα, PPARβ, PPARδ, Foxp3, RORγt, T-bet, IL-17 and IFN-γ are listed below. PPARα: forward primer 5′-GTGCTGTCTATAATTCTGTG-3′, reverse primer 5′-GAAAGTGTCATCTGGATGGTT-3′. PPARβ: forward primer 5′-AGTGACCTGGCGCCTCTCAT-3′, reverse primer 5′-GCCAGATGGTCTGCTCTGAT-3′. PPARδ: forward primer 5′-CATGGCTGTTAAGGATGCAAG-3′, reverse primer 5′-TTCTGAAAACAGACATGTCAT-3′. Foxp3: forward primer 5′-CCCAGAAAGACAGACACTT-3′; reverse primer 5′-TCTTCAAAACCCGGCACCAGTC-3′. RORγt: forward primer 5′-GTCTGGTGTAGGCAGTGGTCAAC-3′, reverse primer 5′-TGCTGAGGAGGCCCTCAGA-3′. T-bet: forward primer 5′-GCAGGAAACCGCTATATG-3′, reverse primer 5′-GACGATCATCTGGTCTACATTG-3′. IL-17: forward primer 5′-GTCGAGGAGGCCCTCAGA-3′, reverse primer 5′-AGCTTCTGGTCCCTGATTA-3′. IFN-γ: forward primer 5′-GCCCTGAGAATGAAGCT-3′; reverse primer 5′-AAAGAGATAATCTGG-3′.

2.4. Detection of cytokines

The CNS-infiltrating CD4 T cells from the MOG35–55-inoculated mice were stimulated by immobilized anti-CD3 antibodies (1 μg/ml) for 48 h in the standard medium in 96-well flat-bottom plates at 0.8 × 10⁶ cells per well on day 16 after the EAE induction. Draining lymph node and spleen cells were cultured in 1640 RPMI medium containing 1% autologous serum with 100 μg/ml MOG35–55 peptides for 48 h. The concentrations of IFN-γ, interleukin-17 and IL-10 in the supernatants were measured by using a sandwich ELISA following the protocol provided by R&D Systems (Minneapolis, MN).

2.5. Statistics

For statistical analysis, non-parametric Mann–Whitney’s U-test or Student’s t test was used. Values of P < 0.05 were considered statistically significant.

3. Results

3.1. EPA substantially ameliorates EAE

To investigate the direct effects of ultra-purified EPA on the pathogenesis of EAE, B6 mice affected with EAE were fed with a fish-oil-free diet without or with 5% (w/w) EPA supplementation 14 days before the EAE induction. The clinical manifestations were evaluated by scoring the severity of EAE, and the histology was assessed by HE and LFB staining of paraffin-embedded spinal cord sections. Notably, the severity of the clinical score in EPA-treated mice was significantly and substantially reduced compared with that of the control mice (Fig. 1a). Spinal cord sections prepared on day 30 also showed reduced infiltrations of mononuclear cells in HE stain and demyelinating foci in LFB stain (Fig. 1b). These results indicated that continuous simple

![Fig. 1. EPA efficiently ameliorated EAE. (a) Two groups of C57BL/6j female mice (n = 5 for each treatment) were fed fish-oil-free diets without or with 5% (w/w) EPA supplementation starting 14 days before inoculation with MOG35–55 peptides emulsified in CFA subcutaneously. Pertussis toxin was injected intra-peritoneally on days 0 and 2. The gray bars indicate administration of EPA; diets were exchanged every morning at 8 o’clock to prevent oxidation. The clinical scores of severity in EAE are indicated as means±SDs (n=5) (* P < 0.05 by Mann–Whitney U test). (b) Histological analysis of spinal cords removed on day 30 after the EAE induction. Sections obtained from cervical cord regions were stained with HE or LFB. Infiltration of mononuclear cells and demyelination of the cervical cord regions were analyzed for EPA-treated and control mice.](image-url)
supplementation of sufficient EPA markedly reduced the infiltration of immune cells into the CNS and inhibited the progress and/or severity of EAE.

3.2. EPA inhibited inflammatory cytokines in CNS-infiltrating CD4 T cells but not those elsewhere

To explore the inhibitory role of EPA on the pathogenesis of EAE, we induced EAE in two groups of four mice each in the same manner as described above and sacrificed the mice on day 16 as indicated (Fig. 2a). Draining lymph node cells and spleen cells were cultured in the 1% autologous serum containing 1640 RPMI medium with 100 μg/ml MOG35-55 peptides at 1×10^6 cells/well for 48 h. Furthermore, the CNS-infiltrating mononuclear cells were harvested and CD4 T cells were purified by magnetic beads. The CNS-infiltrating CD4 T cells were stimulated with immobilized anti-CD3 antibodies for 48 h at 0.8×10^5 cells/well (Fig. 2b). Cytokine levels of IL-17, IFN-γ and IL10 in the supernatants were analyzed by ELISA methods.

The productions of these three cytokines by cells from draining lymph nodes and spleens stimulated by MOG35-55 peptides were not significantly different between the EPA-treated and control mice. The EPA-treated spleen cells had an inclination to increase IL-17 production and decrease IL-10 without statistical significance (Fig. 2a). However, both IFN-γ and IL-17 productions of those CNS-infiltrating mononuclear cells were markedly inhibited in the EPA-treated mice compared with the control mice, consistent with the clinical scores (Fig. 2b). These results indicated that the cytokine levels in the CNS do not correlate with peripheral response, suggesting a selective infiltration of immune cells into the CNS.

3.3. Late-phase supplementation with EPA also ameliorated EAE and augmented PPARs

Next, to examine whether EPA is effective even after immunization by MOG35-55 peptides, we administered EPA 7 days after immunization. The clinical severity of EAE was also markedly reduced even by late-phase administration of EPA compared with the control mice (Fig. 3a). EPA is a natural ligand of three types of PPARs, PPARα, β, and γ (Xu et al., 1999), and these PPARs have been reported to play inhibitory roles on the pathogenesis of EAE (Dunn et al., 2007; Klotz et al., 2009; Dunn et al., 2010). Therefore to explore if the possible role of anti-inflammatory effect of EPA is dependent on PPARs induction, we...
investigated whether EPA induced PPARs in the CNS-infiltrating CD4 T cells. After the EAE induction mice were sacrificed on day 17, the CNS-infiltrating mononuclear cells were harvested and CD4 T cells were purified from the CNS-infiltrating cells by magnetic beads. Transcriptional levels of PPARs in purified CNS-infiltrating CD4 T cells were measured by quantitative RT-PCR. The expression levels of all PPARs were enhanced significantly in the EPA-treated mice (Fig. 3b). These results indicated that EPA induced PPARs efficiently in the CNS-infiltrating CD4 T cells and the supplementation of EPA after the EAE induction still had anti-inflammatory effects on the development of EAE.

3.4. EPA did not effectively augment PPARs but promoted Treg/Th17 differentiation in the periphery

To evaluate the peripheral effect of EPA on PPARs induction, we also measured the value of PPARs in spleen CD4 T cells obtained on day 14 from MOG35–55 peptide-inoculated mice without PT injection by using real-time PCR. Only PPARγ was induced weakly in the spleen CD4 T cells, but the inductions of PPARα and PPARβ showed no difference between the EPA-treated and control mice (Fig. 4a). These results may indicate that EPA can induce PPARs mainly on the CNS-infiltrating CD4 T cells rather than on the peripheral CD4 T cells and that plays an inhibitory role.

Furthermore, we determined the transcriptional levels of master genes of regulatory T cells (Treg), Th17 cells and Th1 cells, and cytokine levels of IL-17 and IFN-γ at the same time. As shown in Fig. 4b, Foxp3 and RORγt were increased simultaneously with the augmentation of IL-17. Treg cells and Th17 cells are typically expected to differentiate reciprocally. Therefore, this may indicate that EPA could induce FoxP3-positive regulatory T cells effectively in the spleen but could not inhibit their conversion into IL-17-producing regulatory T cells in this method of supplementation.

4. Discussion

A previous report several decades ago suggested the clinical benefits of n−3 PUFA patients with MS (Bates et al., 1989) and a more recent report demonstrated that a low-fat diet with n−3 PUFAs significantly reduced the relapse rate in relapse remitting MS patients (Weinstock-Guttman et al., 2005). Conversely, there is a report that n−3 PUFAs as a monotherapy or in combination with interferon beta-1α had no beneficial clinical effects on the disease activity including magnetic resonance imaging (MRI) activities (Torkildsen et al., 2012). Therefore, the clinical effects of n−3 PUFA are still controversial.

In this context, we conducted a series of experiments to verify the therapeutic potential and to explore the mechanism of EPA on the pathogenesis of EAE/MS. Contrary to our expectations for these clinical trials, the therapeutic effects of ultra-purified EPA on the development of EAE were powerful. EPA clearly reduced the severity of clinical scores and the CNS-infiltrating cells were reduced in pathological histology in the EPA-treated mice. Additionally, the administration of EPA 7 days after immunization still significantly ameliorated EAE. According to the previous reports using atherosclerosis models, EPA inhibits the expression of the adhesion molecules of endothelial cells, ICAM-1, VCAM-1,
E-selectins, etc. (Matsumoto et al., 2008), and the local induction of endothelial VCAM-1 and ICAM-1 around adhering T cells as a prerequisite in the CNS is required for their subsequent extravasation (Xu et al., 2003). Therefore we speculate that the reduced numbers of the CNS infiltrating cells in EPA treatment might be due to the inhibition of the up-regulation of adhesion molecules on endothelial cells of the CNS by inhibiting the production of inflammatory eicosanoids.

Furthermore, our data clearly showed intriguing results that EPA induced all types of PPARs and almost completely blocked the production of inflammatory cytokine in the CNS-infiltrating CD4 T cells. According to previous studies, PPARγ agonists repressed the Th1 responses and reduced the severity of EAE in MOG35-55 immunized mice and this advantage was clearly reversely by using PPARγ antagonists, bisphenol A diglycidyl ether (BAGDE) and 2-chloro-5-nitro-N-(4-pyridyl)benzamide (T0070907), restoring T cell proliferation and the Th1 responses inhibited by PPARγ agonists (Raikwar et al., 2006). A more recent report showed that PPARγ also inhibited the Th17 differentiation and the production of IL-17 in a manner intrinsic to T cells (Klotz et al., 2009). On the other hand, PPARα directly binds to nuclear factor ρB (NF-κB) and c-jun and represses the DNA-binding of NF-κB and c-jun ligand-dependently, leading to a reduced production of IFN-γ from T cells (Dunn et al., 2007). Moreover, PPARs inhibit IL-17 and IFN-γ production and differentiation into cytokine-producing CD4 T cells in response to stimulation (Dunn et al., 2010). Besides those in vitro anti-inflammatory mechanisms, PPARα- and β-selective agonists ameliorate EAE, and conversely, PPARs- and δ-deficient mice each develop more severe EAE. Therefore, we speculate that inductions of PPARs in the CNS-infiltrating CD4 T cells lead to the inhibition of IL-17 and IFN-γ cytokine productions.

The blood–brain barrier (BBB) is a support system for the immune privilege of the CNS. In fact, mononuclear cells constitutively penetrate endothelial tight junctions through the trans-cellular pathway and accumulate in the perivascular space, but they do not pass the glia limitans (Bechmann et al., 2007). Myelin antigen-specific auto-reactive CD4 T cells are known to be re-stimulated in the perivascular space of the CNS (Ouellet et al., 2009). Myelination of the CNS occurs continuously throughout the lifespan and is responsible for the maintenance of the BBB. Therefore, we speculate that the tendency of reactivity of BBB enables the supplemented EPA to induce all types of PPARs in the CNS in a transitional stage in which we need to assess the potentially separate effects of EPA and DHA instead of the combination therapy in metabolic diseases as well as multiple sclerosis.

Supplementation of n-3 PUFAs leads to incorporation into the plasma membrane as phospholipids, and cytosolic phospholipase A2 (cPLA2) releases mainly AA and EPA but rarely DHA from phospholipids by various stimulations. Released EPA is converted into docosapentaenoic acid (DPA, 22:5) by endogenous elongase, and DPA inhibits cyclooxygenase, leading to reduced production of PGE2 derived from AA (Norris and Dennis, 2012). Besides that, supplementations by EPA and DPA, but not DHA, are associated with lower risk of nonfatal cardiovascular endpoints in some studies, and purified EPA reduced the risk of nonfatal coronary syndromes in one large clinical trial (Mozaffarian and Wu, 2012). Those findings indicate that the roles of EPA and DHA are not entirely the same. A clinical trial of n-3 PUFAs supplementation resulted in a considerable outcome using the combination of EPA and DHA, but there has been no report about supplementation with purified-EPA alone. Ultra-purified EPA of 98% purity, has become available in Japan ahead of the rest of the world and is producing good clinical outcomes in the prevention of cardiovascular attacks (Yokoyama et al., 2007). Therefore, we are in a transitional stage in which we need to assess the potentially separate effects of EPA and DHA instead of the combination therapy in metabolic diseases as well as multiple sclerosis.

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References


Beriou, G., Costantino, C.M., Ashley, C.W., Yang, L., Kuchroo, V.K., Baecker-Allan, C., Hafler, D.A., 2009. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 113, 4240–4249.

Cawood, A.L., Ding, R., Napper, F.L., Young, R.H., Williams, J.A., Ward, M.J., Gudmundsen, O., Vige, R., Payne, S.P., Ye, S., Shearman, C.P., Gallagher, P.J., Grimbale, R.F., Calder, P.C., 2010. Eicosapentaenoic acid (EPA) from highly concentrated n-3 fatty acid ethyl esters is incorporated into advanced atherosclerotic plaques and higher plaque EPA is associated with decreased plaque inflammation and increased stabili-


ome proliferator-activated receptor delta limits the expansion of pathogenic Th cells during central nervous system autoimmunity. J. Exp. Med. 207, 1599–1608.

Gold, R., Linsington, C., Lassmann, H., 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimen-


Haworth, O., Cernadas, M., Yang, R., Serhan, C.N., Levy, B.D., 2008. Resolvin E1 regulates interleukin 23, interferon-gamma and lipoxin A4 to promote the resolution of aller-


